CHAPTER III

RESULTS

3.1 Characterization of monoclonal antibodies against hemoglobins

Various hybrid clones producing anti-hemoglobin mAbs were provided by Assoc. Prof. Dr. Watchara Kasinrerk. These mAbs were produced from BALB/C mice immunized with Hb Bart's hydrops fetalis hemolysate by a standard hybridoma technique. The hybridoma clones producing anti-hemoglobin mAbs were screened by indirect ELISA. As shown in Figure 3.1, two hybridoma clones named Thal GJA and Thal N/B were of interested. The Thal GJA clone produced mAb that strongly reacted with only Hb Bart's hydrops fetalis hemolysate which contain 90% of Hb Bart's. MAb Thal N/B was strongly positive with all hemolysate tested including Hb Bart's hydrops fetalis hemolysate, normal cord blood hemolysate and normal adult hemolysate. However, the reactivity to normal hemolysate was weaker compare to other hemolysate antigens (Figure 3.1).

Mabs Thal GJA and Thal N/B were subjected for limiting dilution technique to get single clone. The culture supernatant from the cloned hybridomas were collected and re-tested for its specificity. After that the Thal GJA and Thal N/B hybridoma clones were injected to produce ascitic fluid in mice. The ascitic fluids containing anti-Hb Bart's or anti-Hb mAbs were purified by AKTA prime using Hitrap Protein G affinity columns.

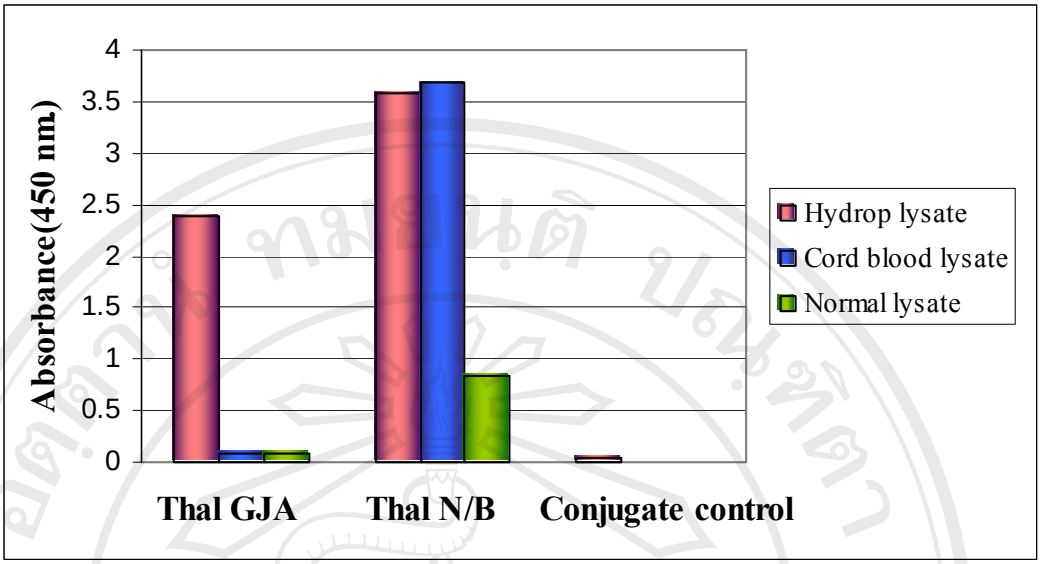


Figure 3.1 Determination of the specificity of Thal GJA and Thal N/B monoclonal antibodies. The 96-well plate was coated with indicated hemolysates, including Hb Bart's hydrops fetalis hemolysate, cord blood hemolysate and normal adult hemolysate. The mAbs Thal GJA and Thal N/B activity was determined by indirect ELISA. The reaction was measured by spectrophotometer at O.D. 450 nm.

3.2 Determination the activity and specificity of purified monoclonal antibodies against hemoglobins

To verify the activity and specificity of the purified mAbs, which obtained from affinity chromatography, a set of indirect ELISA was performed. In this study, 50 μg/ml of Hb Bart's hydrops fetalis hemolysate, normal cord blood or normal blood hemolysate was coated into 96-well plate and incubated at 4°C overnight, then 20 μg/ml of purified mAbs was added following by adding HRP-rabbit anti-mouse immunoglobulins antibodies. The results confirmed that mAb Thal GJA was strongly positive with Hb Bart's hydrops fetalis hemolysate, but did not react to normal and cord blood hemolysates. However, purified Thal N/B strongly reacted to hemolysate of Hb Bart's hydrops fetalis and cord blood, but weakly reacted to normal hemolysate as shown in Figure 3.2. These results indicated that the purified mAbs could be used in the further experiments.

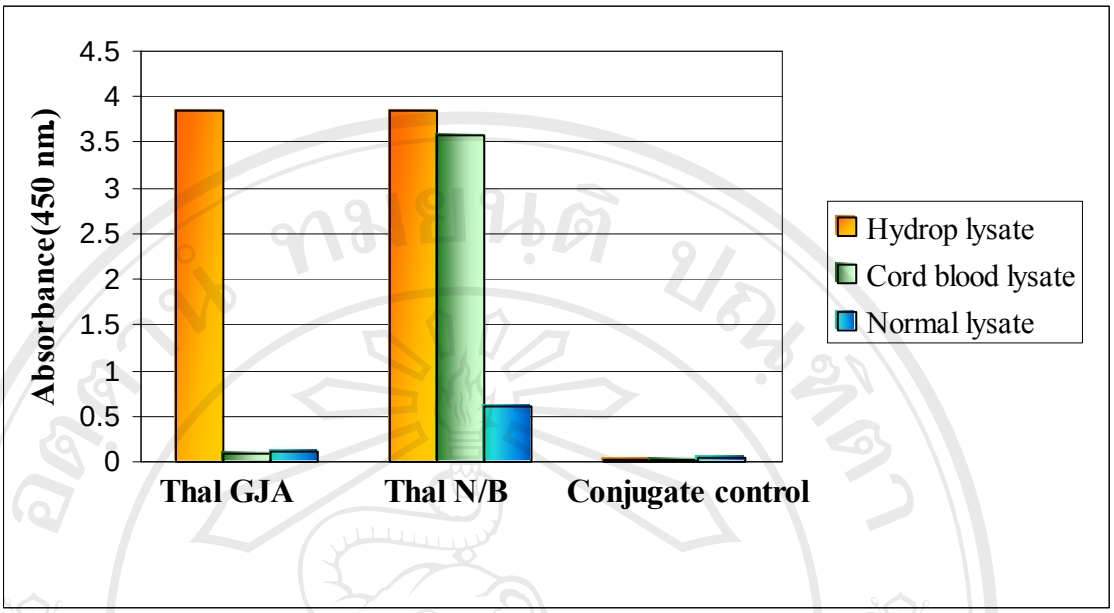


Figure 3.2 Specificity of purified mAbs Thal GJA and Thal N/B by indirect ELISA. Various hemolysates were coated into 96-well plate. Then 20 µg/ml of purified mAbs Thal GJA and Thal N/B were added following by HRP conjugated rabbit anti-mouse immunoglobulins antibodies. The reaction was measured by spectrophotometer at O.D. 450 nm.

3.3 Detection of hemoglobin Bart's in blood samples by Sandwich ELISA

3.3.1 Preparation of HRP conjugated mAb Thal N/B

To develop sandwich ELISA for detection of Hb Bart's in blood sample, enzyme conjugate anti-hemoglobin mAb was required. In this study, we labeled HRP to mAb Thal N/B. Indirect ELISA was performed to determine the activity of the labeled mAb. In this study, 96-well plate was coated with 50 μg/ml of Hb Bart's hydrops fetalis hemolysate, then Thal N/B-HRP or Thal N/B-HRP preserved in sodium aizde (NaN_3) at various concentrations were added. The activity of HRP labeled Thal N/B was determined at O.D. 450 nm. As shown in Figure 3.3, both HRP-labeled Thal N/B with or without preservative (NaN_3) showed high activity and sensitivity at concentration of 10 ng/ml. This experiment indicated that NaN_3 can be used as preservative for HRP conjugate.

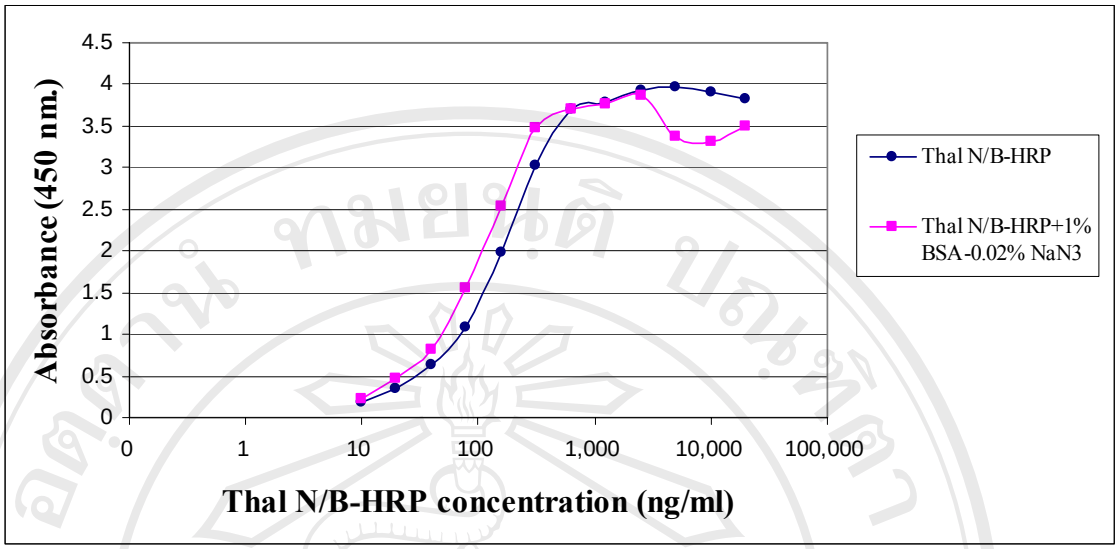


Figure 3.3 Activity of HRP-conjugated purified mAb Thal N/B in the presence or absence of sodium azide preservative. 50 µg/ml of Hb Bart's hydrops fetalis hemolysate were coated on 96-well plate. HRP labeled Thal N/B or HRP labeled Thal N/B preserved in NaN_3 at various concentrations were added. The reaction was measured by spectrophotometer at O.D. 450 nm.

3.3.2 Development of method for determination of hemoglobin Bart's by sandwich ELISA using mAbs Thal GJA and HRP-labeled Thal N/B

In order to set up a simple immunoassay for quantification of Hb Bart's in hemolysate, the sandwich ELISA was developed. Various concentrations of Thal GJA anti-Hb Bart's mAb were coated into 96-well plate. Thereafter various concentrations of Hb Bart's hydrops fetalis hemolysate, normal cord blood hemolysate and normal hemolysate was added following by adding HRP-conjugated Thal N/B anti-Hbs mAb. The results demonstrated that the positive reactivity with Hb Bart's hydrops fetalis hemolysate was correlated to the increased concentrations of Thal GJA and HRP-labeled Thal N/B as shown in Figure 3.4. Normal cord blood hemolysate and normal hemolysate showed very slightly reactivity.

The mAb Thal GJA at concentration 100 and 200 μg/ml and HRP-labeled Thal N/B at 0.625 and 1.25, μg/ml were selected for study in detail. As shown in Figure 3.5, the optimal condition which showed highest sensitivity in detection of Hb Bart's was as follows: Thal GJA anti-Hb Bart's mAb at 100 μg/ml was used as a capture antibody and HRP-conjugated Thal N/B anti-Hbs mAb at 1.25 μg/ml was used as detecting antibody. At this condition, no positive reactivity with cord blood and normal hemolysate was observed. Therefore, these concentrations were selected for further studies.

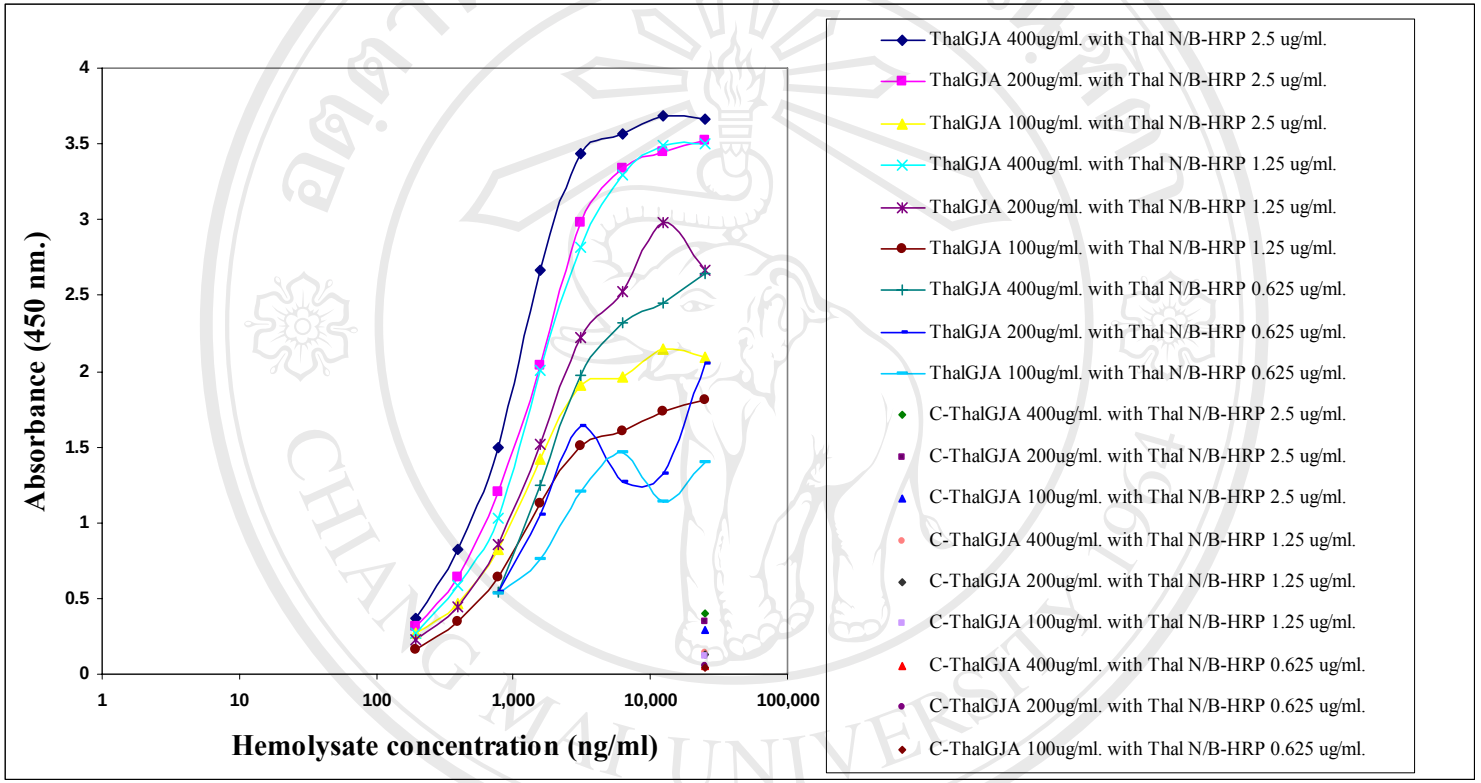


Figure 3.4 The optimal concentration of Thal GJA and HRP-conjugated Thal N/B mAb for detection of Hb Bart's in hemolysate by developed sandwich ELISA. The 96-well plate was coated with various concentrations of Thal GJA anti-Hb Bart's mAb. Various concentrations of Hb Bart's hydrops fetalis hemolysate, cord blood hemolysates and normal hemolysate were added. The Hb Bart's concentration was detected by using HRP-conjugated Thal N/B as a conjugate.

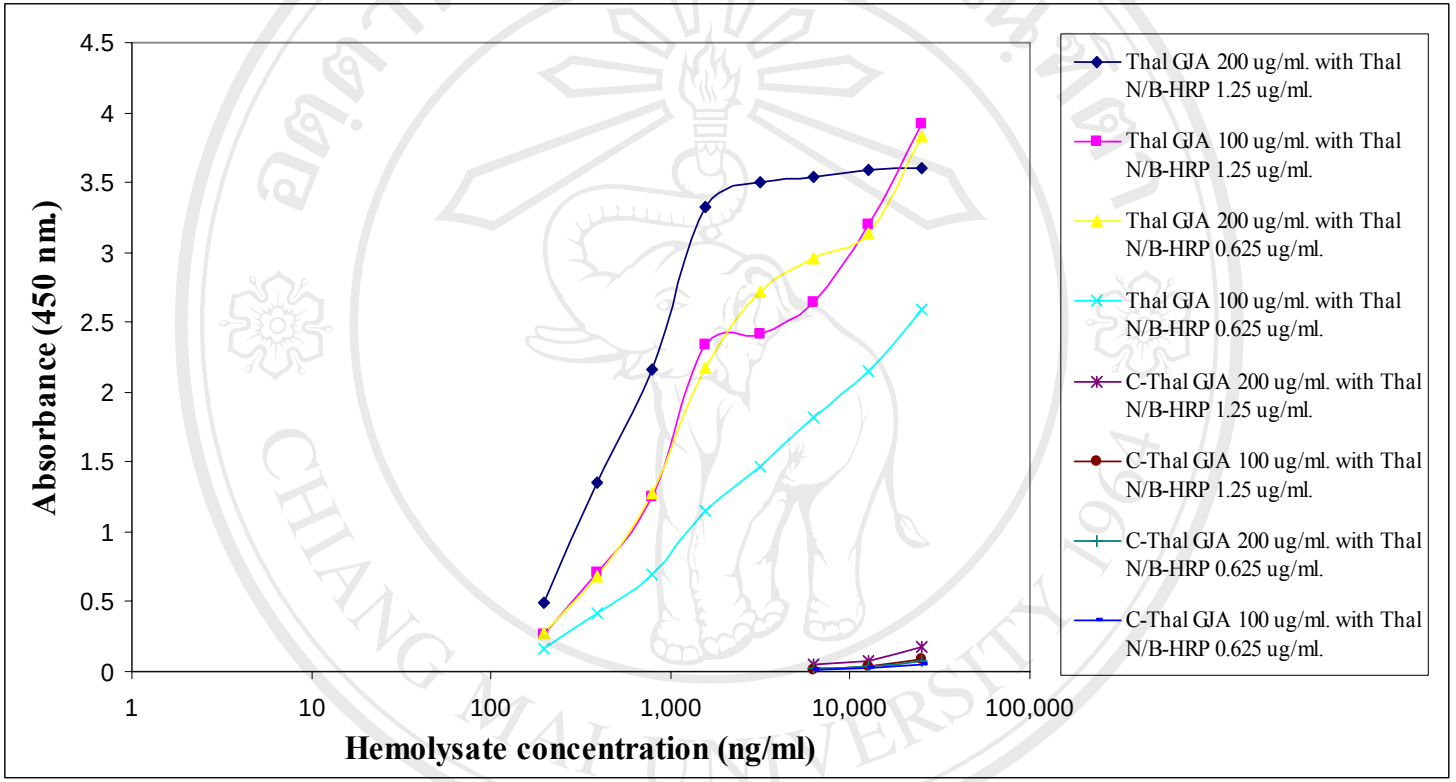


Figure 3.5 The optimal concentration of Thal GJA and Thal N/B-HRP to detect Hb Bart's in hemolysate was determined. Plate was coated with 100 or 200 µg/ml of Thal GJA. Then, Hb Bart's hydrops fetalis hemolysate, cord blood and normal hemolysates were added. The Hb Bart's concentration was detected after adding HRP-conjugated Thal N/B at 0.625 or 1.25 µg/ml.

3.4 Determination of specificity and sensitivity of hemoglobin Bart's of the developed sandwich ELISA

To determine the specificity and sensitivity of Hb Bart's of the developed sandwich ELISA, the purified Thal GJA at concentration 100 μg/ml was coated onto 96-well plate. Then purified Hb Bart's, Hb F, Hb A, Hb A_2, and Hb E at various concentrations were added. The HRP-labeled Thal N/B at concentration 1.25 μg/ml was used as detecting antibody. As shown in Figure 3.6, only purified Hb Bart's was captured by Thal GJA anti-Bart's mAb and detected by HRP-conjugated Thal N/B anti-Hbs mAb. The sensitivity of sandwich ELISA for determining Hb Bart's was 10 ng/ml The developed sandwich ELISA was then used for screening Hb Bart's in the thalassemia samples.

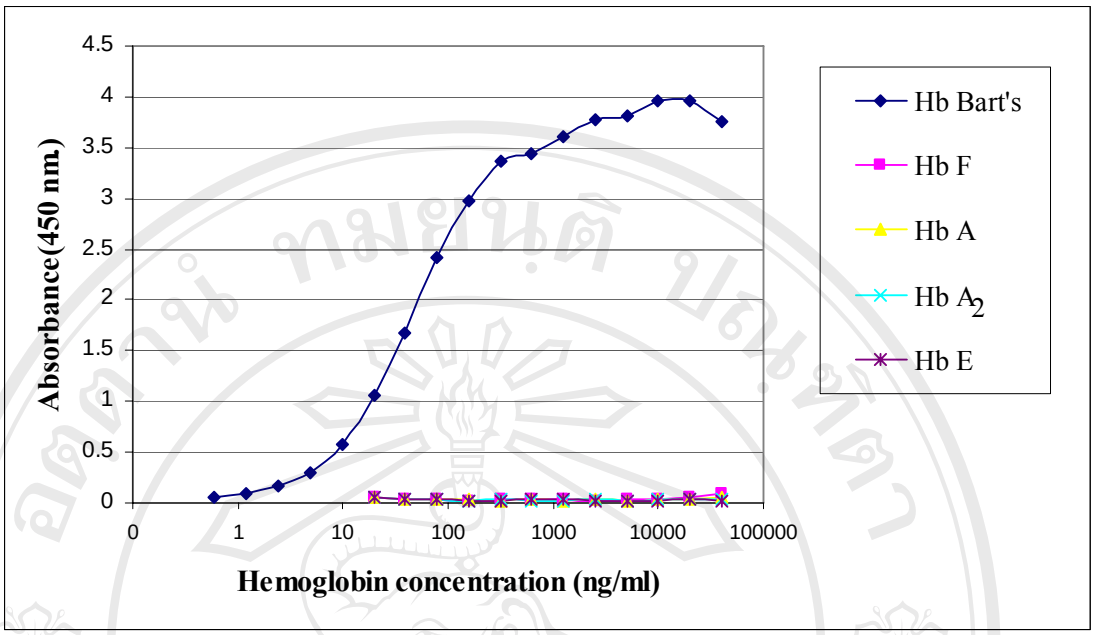


Figure 3.6 Specificity and sensitivity of the developed sandwich ELISA. The Thal GJA anti-Hb Bart's mAb was coated on 96 well plates. Then, various concentrations of Hb Bart's, Hb F, Hb A, Hb A_2 and Hb E were added. The reactivity was detected after adding HRP-Thal N/B anti-Hbs mAb and measured at O.D. 450 nm.

3.5 Evaluation of the developed sandwich ELISA for quantification of hemoglobin Bart's in thalassemia hemolysate

3.5.1 Screening for thalassemia heterozygotes

3.5.1.1 Blood samples

A total of 91 blood samples were deliberately selected from the Clinical Microscopy Unit, Department of Central Laboratory, Maharaj Nakorn Chiang Mai Hospital. The primary criterion for selecting blood samples comprised MCV value of less than 80 fL and hemoglobin (Hb) concentration more than 12 g/dl. From total 91 subjects, 60 blood samples with Hb concentration of 12.7 ± 1.0 g/dl (mean ± SD) were suspected to be thalassemia heterozygote, whereas 31 cases with normal MCV and normal Hb concentration were used as control group.

3.5.1.2 One-tube osmotic fragility test (OF test)

The OF test was used for preliminary screening of α-thalassemia and β-thalassemia. The test was done as follows: 15 μl of EDTA-blood was mixed well with 2 ml of 0.36% saline buffer. After standing at room temperature for 15 min, the OF test was visualized to consider positive or negative. Negative samples were characterized by a clear red hemoglobin solution indicating the RBC was completely hemolyzed, whereas positive samples were identified by a cloudy appearance because of incomplete hemolysis. The result found that 56 out of 91 blood samples were positive with OF test whereas 35 samples were OF test negative.

3.5.1.3 Hb H inclusion body test

To screen α-thalassemia from blood samples, the Hb H inclusion body test using the modified BCB method was performed with 60 blood samples with abnormal CBC. Within the 60 blood sample tested, 56 blood samples were positive with OF test and 4 samples were negative. As shown in Figure 3.7, the Hb H inclusion body was screened in 10,000 RBCs under light microscope. Each blood sample was done in duplicate. It was found that 40 out of 60 blood samples were positive with Hb H inclusion body while 18 samples were negative and 2 samples were indeterminate because of unqualified slide preparation (data not shown).

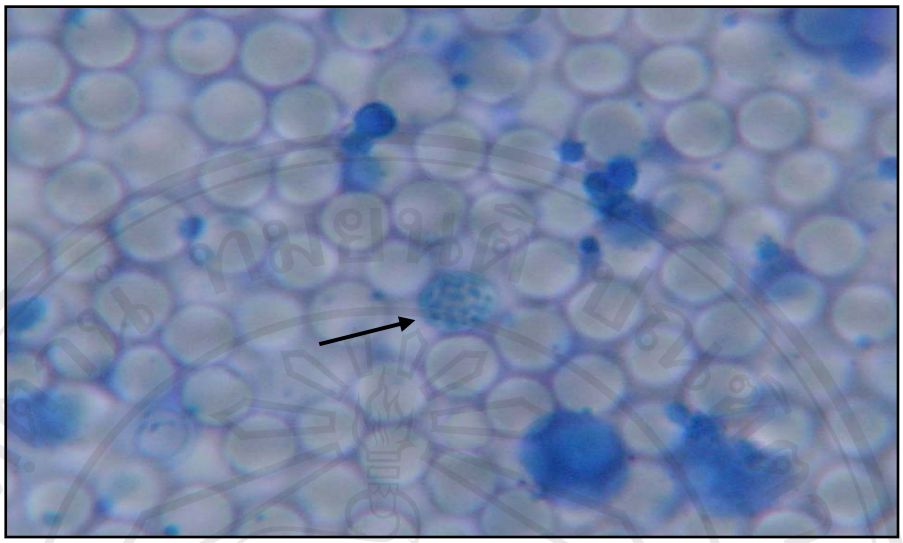
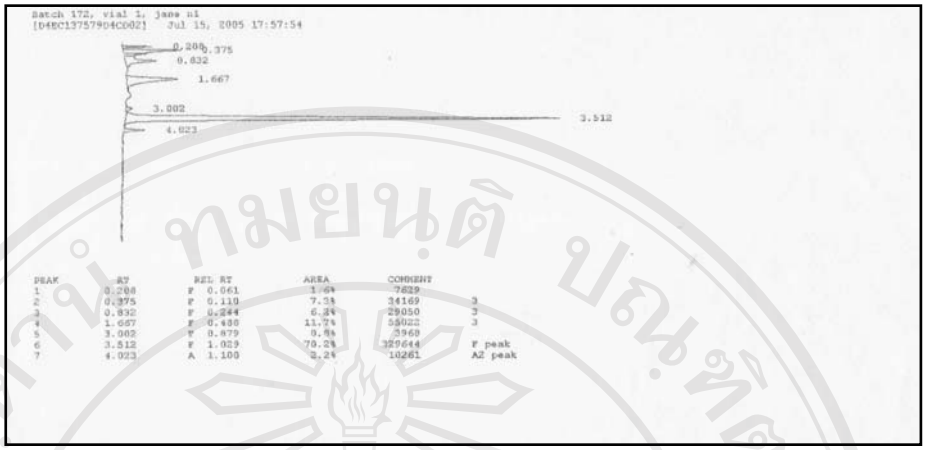


Figure 3.7 Hb H inclusion bodies screening in α-thalassemia 1 heterozygote by modified BCB method. Blood samples were incubated on the slide coated with dried brilliant cresyl blue at room temperature for overnight. The Hb H inclusion body was screened in 10,000 RBCs under light microscope. The arrow indicated red blood cell containing Hb H inclusions.

3.5.1.4 High Performance Liquid Chromatography analysis for β-thalassemia

To screen for heterozygous state of β hemoglobinopathies, the weak-cation exchange HPLC analysis was carried out in 91 blood samples as mentioned previously in Materials and Methods. Normal Hb typing (A_2A) with normal Hb A_2 level (2.2 ± 0.6; mean ± 2SD) was demonstrated in 31 blood samples with normal CBC results. On the other hand, in the rest 60 blood samples, 19 were characterized as β-thalassemia heterozygote (A_2A, Hb A_2 6.5 ± 1.9; mean ± SD), 15 as Hb E heterozygote (AE, Hb E 20.3 ± 8.0; mean ± SD) as shown in Figure 3.8. The remaining 26 blood samples had normal Hb typing as well as normal Hb A_2 level and subsequently found to be α-thalassemia 1 heterozygote by the Gap-PCR technique.

A



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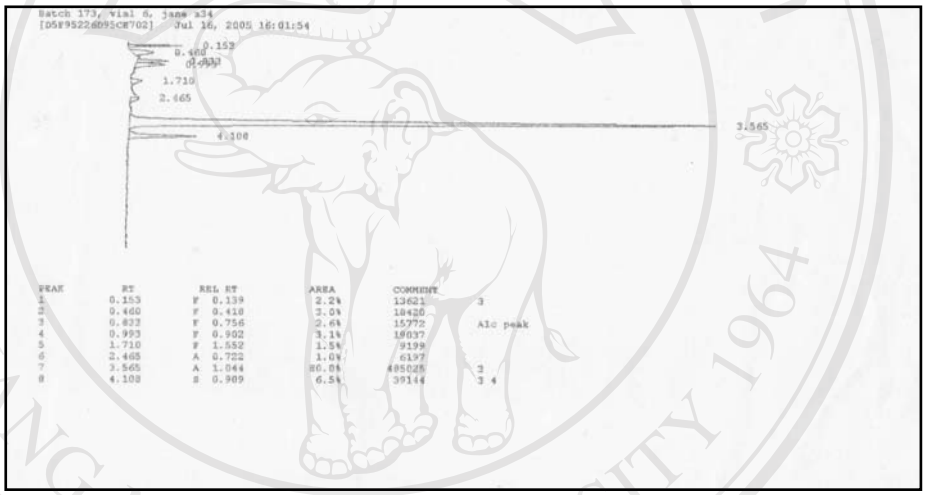

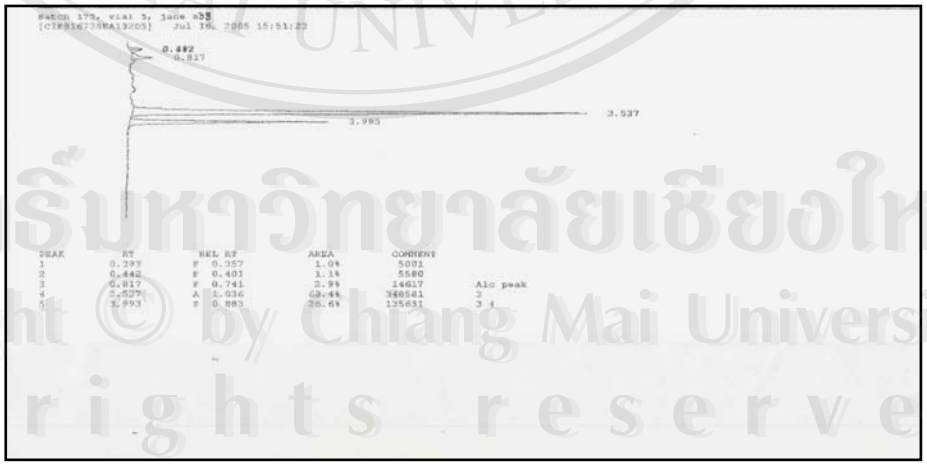

Figure 3.8 Three representative types of HPLC chromatograms from blood samples. (A) Normal genotype, (B) β-thalassemia heterozygote, (C) Hb E heterozygote.

3.5.1.5 PCR analysis for α-thalassemia

After thalassemia screening was processed, all whole blood samples were divided into two vials for detection of Hb Bart's by the developed sandwich ELISA and for PCR analysis.

For sandwich ELISA, the hemolysate was prepared from 500 μl of whole blood. The blood was centrifuged at 2000g, 4°C for 10 min and plasma was discarded. Packed red blood cells was washed with normal saline and then lysed by distilled water plus toluene. The clear hemolysate was transferred in new vial and stored at -20°C.

For PCR analysis, DNA was extracted from the buffy coat using standard phenol-chloroform technique.

3.5.1.5.1 Detection α-thalassemia 1 with the Southeast Asian deletion (SEA type) by using Gap-PCR analysis

To detect α-thalassemia 1 with SEA type, the Gap-PCR analysis was performed in 60 blood samples with abnormal CBC (MCV in range of 63-79 fL). After PCR process, the amplified product was run on 2.5 % agarose gel eletrophoresis and stained with ethidium bromide. The gel was visualized under UV transilluminator. As shown in the Figure 3.9, the 314 bp fragment, same as that observed in the internal control, was observed in all samples. For α-thalassemia1 heterozygote (SEA type), a band at 188 bp was found.

Among 60 blood samples with abnormal CBC, it was found that 26 samples were identified as α-thalassemia 1 with SEA deletion, 6 samples were detected as double heterozygotes of α-thalassemia 1 with SEA deletion and β-thalassemia and 6

samples were double heterozygotes of α-thalassemia 1 (SEA deletion) and Hb E. Another 22 samples were negative for α-thalassemia 1 with SEA deletion.

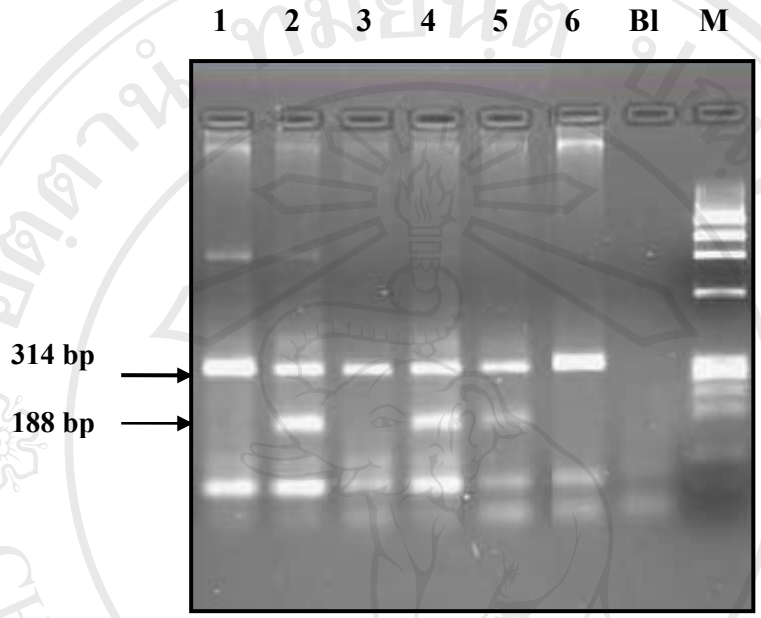

Figure 3.9 PCR analyses for α-thalassemia 1 Southeast Asian deletion type by agarose gel electrophoresis and stained with ethidium bromide. The results demonstrated that the PCR products were produced with only 314 bp for normal α-globin gene using P1 and P2 primer (Lane 1, 3 and 6). Whereas fragments with 314 bp and 188 bp using P1 and P3 primer was observed in α-thalassemia 1 SEA type (Lane 2, 4 and 5). Bl and M were represented as blank control and øX174 HaeIII digested DNA size markers, respectively.

3.5.1.5.2 Detection α-thalassemia 2 with 3.7 kb and 4.2 kb deletions by PCR analysis

In order to identify homozygous and heterozygous of α-thalassemia 2 with 3.7 kb or 4.2 kb deletion, PCR analysis was also performed. The primer A and primer B was used to amplify a 1779 bp specific fragment which specific for α-thalassemia 2 with 3.7 kb deletion (Figure 3.10). The primer C and primer D was used to amplify a 1529 bp fragment in α-thalassemia 2 with 4.2 kb deletion (Figure 3.11) and primer αG1 and αG11 was used to amplify a 1395 bp fragment for α-globin gene with no deletion.

Identification of α-thalassemia 2 with 3.7 kb and 4.2 kb deletions was carried out in 53 blood samples. Among of them, 31 samples have normal CBC profile and 22 samples were negative for α-thalassemia 1 with SEA deletion.

It was found that the most common α-thalassemia 2 with 3.7 kb deletion was identified in 4 of the 31 samples with normal CBC. No α-thalassemia 2 with 4.2 kb deletion was found among those 31 samples. Among 22 samples with negative for α-thalassemia 1 with SEA deletion, 5 samples were found to be double heterozygotes of α-thalassemia 2 (3.7 kb deletion) and β-thalassemia. One sample was identified as double heterozygotes of α-thalassemia 2 with 3.7 kb and Hb E. Furthermore, one sample was identified as double heterozygotes of α-thalassemia 2 with 4.2 kb deletion and Hb E.

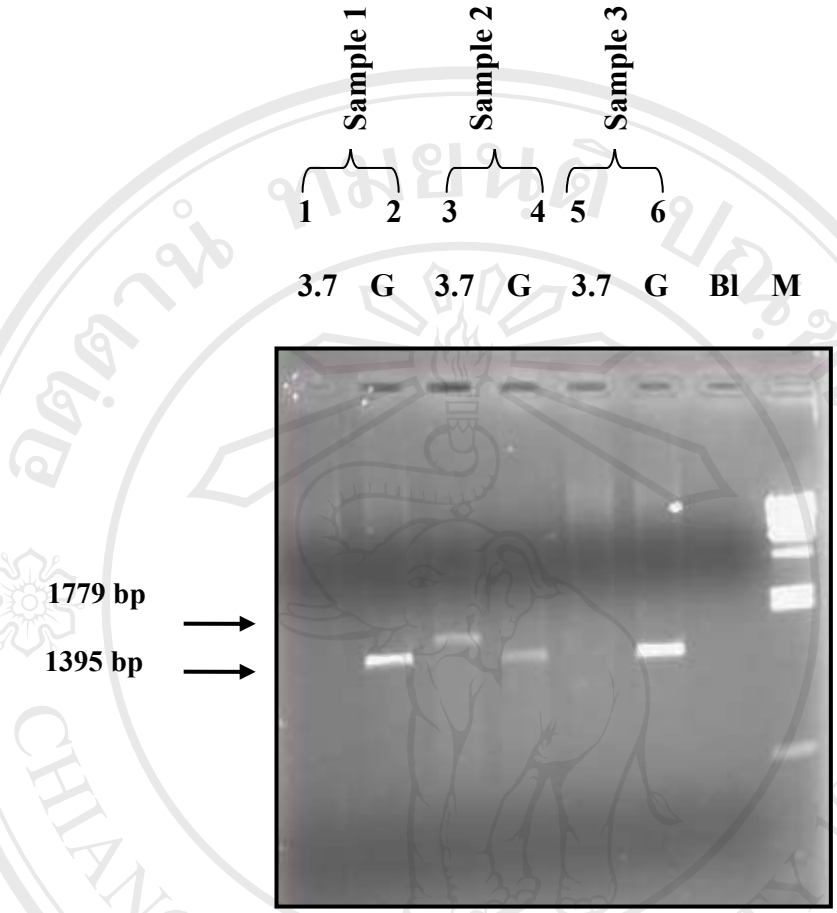

Figure 3.10 PCR analyses for α-thalassemia 2 with 3.7 kb deletions by agarose gel electrophoresis. The amplified PCR products from three different samples were demonstrated. The fragment with 1395 bp using αG1 and αG11 primer was detected in all three samples (Lane 2, 4 and 6) as an internal control. Whereas fragments with 1779 bp indicating α-thalassemia 2 heterozygote with the 3.7 kb deletion was observed in sample 2 (Lane 3). Bl and M were represented as blank control and λ – DNA/Hind III size markers, respectively.

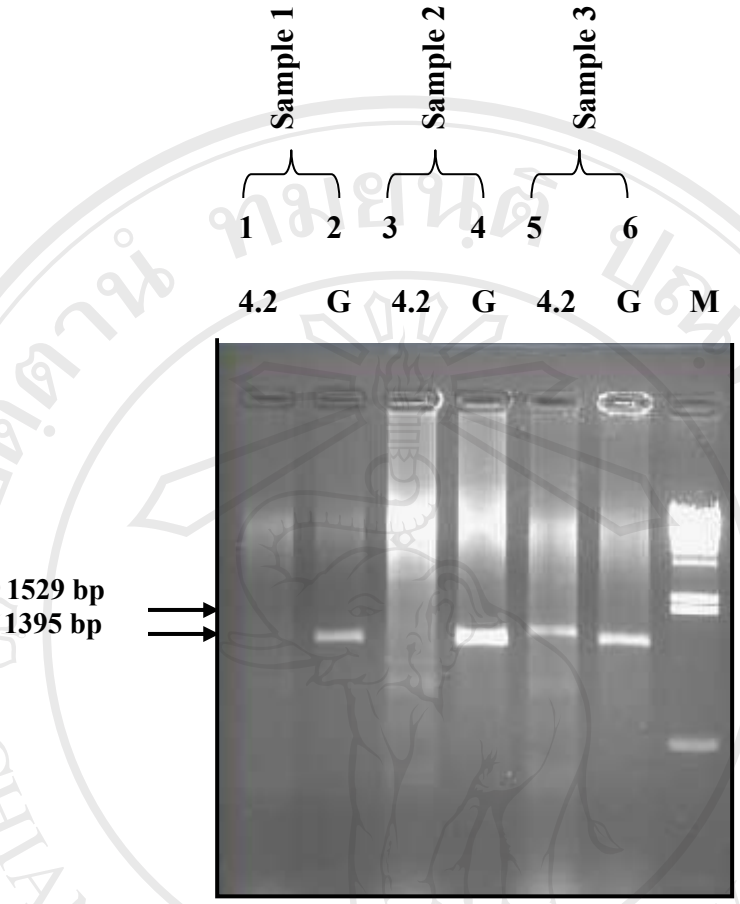

Figure 3.11 PCR analyses for α-thalassemia 2 with 4.2 kb deletions by agarose gel electrophoresis. The fragment with 1395 bp using αG1 and αG11 primer was detected in all three samples (Lane 2, 4 and 6) as an internal control. Whereas fragments with 1529 bp indicating α-thalassemia 2 heterozygote with the 4.2 kb deletion was observed in only sample 3 (Lane 5). Bl and M were represented as blank control and λ –DNA/Hind III size markers, respectively.

3.5.1.6 Comparison of the one-tube osmotic fragility test and PCR test

Of the 56 subjects with a positive OF test, 26 subjects were α-thalassemia 1 heterozygote (SEA type). Six subjects were double heterozygotes of β-thalassemia and Southeast Asian type, 5 subjects were double heterozygotes of α-thalassemia 1 (SEA type) and Hb E. Double heterozygotes of β-thalassemia and α-thalassemia 2 and double heterozygotes of Hb E and α-thalassemia 2 were found in 4 and 2 subjects, respectively. In addition, 8 subjects of β-thalassemia heterozygote and 5 subjects of Hb E heterozygote were positive with OF test.

Among 35 samples with OF test was negative, one sample was double heterozygotes of α-thalassemia 1 (SEA type) and Hb E and double heterozygotes of β-thalassemia and α-thalassemia 2. Two subjects were Hb E heterozygote and four subjects with normal RBC indicies found to be α-thalassemia 2 heterozygote as shown in Table 3.1.

Table 3.1 Screening of 91 blood samples by OF Test using 0.36% buffered saline solution compared with PCR analysis

Classification by PCR analysis	OF Screening	
	Positive (N=56)	Negative (N=35)
Normal	0	27
α-thalassemia 1(SEA type)	26	0
β-thalassemia heterozygote/SEA	6	0
Hb E heterozygote/SEA	5	1
β-thalassemia heterozygote/α-thalassemia 2	4	1
Hb E heterozygote/α-thalassemia 2	2	0
β-thalassemia heterozygote	8	0
Hb E heterozygote	5	2
α-thalassemia 2	0	4

3.5.1.7 Quantitation of hemoglobin Bart's by the developed sandwich ELISA

According to genotyping and phenotyping results, 91 blood samples were divided into 9 groups as shown in Table 3.4.

In this study, a sandwich ELISA for Hb Bart's detection was established using Thal GJA anti-Hb Bart's and Thal N/B anti-Hbs mAb. Thal GJA anti-Hb Bart's mAb was used as a capture antibody and HRP-labeled Thal N/B anti-Hbs mAb was used as a detection antibody. The developed ELISA was then used to determine concentration of Hb Bart's of all 91 samples. We aim to use this developed ELISA for identification of α-thalassemia 1 carriers.

By the developed ELISA, the results showed that Hb Bart's concentrations in α-thalassemia group was higher than β-thalassemia heterozygote or Hb E heterozygote, especially normal group. In group 2, the results demonstrated that 24 samples with α-thalassemia 1 (SEA type) had Hb Bart's concentrations between 6.21 μg/ml to 125.57 μg/ml. Whereas, the Hb Bart's concentrations were detected between 4.50 μg/ml to 156.2 μg/ml in sample group 3 which are double heterozygotes of β-thalassemia and α-thalassemia 1 with SEA deletion. Among 6 samples of double heterozygotes of Hb E with SEA deletion (group 4), they had Hb Bart's concentrations of 8.81 to 36.92 μg/ml. Four samples with double heterozygotes of β-thalassemia and α-thalassemia 2 (3.7 kb deletion) had Hb Bart's concentrations between 4.55 μg/ml to 84.44 μg/ml (group 5). There was one sample with double heterozygotes of Hb E and α-thalassemia 2 and α-thalassemia 2 heterozygote in which Hb Bart's concentrations was 83.21 μg/ml and 6.41 μg/ml, respectively (group 6 and group 9). The remaining 34 samples were normal, β-thalassemia heterozygote

and Hb E heterozygote in which Hb Bart's concentrations was less than 4.3 μg/ml (group 1, 7 and 8). The results were showed in Table 3.2 and Figure 3.13. When Hb Bart's concentrations of samples in each group were analyzed, as was shown in Table 3.2, the Hb Bart's concentrations in group 2 and 3 were higher than in other α-thalassemia heterozygote categories whereas the lowest of Hb Bart's concentrations was normal control group.

Interestingly, the Hb Bart's was detected in 2 normal samples. Furthermore, 4 samples of β-thalassemia and 2 samples of Hb E heterozygote were positive by developed sandwich ELISA method.

The correlation between Hb Bart's concentrations and absorbance at 450 nm of the developed ELISA was analyzed by linear regression model using sigma plot software. In this study, mean ± SD of Hb Bart's concentrations from 25 normal samples were 3.40 ± 0.44. The confidential interval was between 2.52 to 4.28, 95% CI (2.52-4.28). Therefore, Hb Bart's concentration was cut off at 4.5 μg/ml to exclude α-thalassemia 1 heterozygote from other groups.

Table 3.2 The results of developed sandwich ELISA for Hb Bart's in different categories

Category	Method		Hb Bart's concentrations by developed ELISA (μg/ml)
	Standard (PCR)	Developed (ELISA)	
1. Normal	27	25	<4.2
2. α-thalassemia 1 (SEA type)	26	24	3.21 - 125.57
3. β-thalassemia heterozygote/SEA	6	6	4.50 - 156.2
4. Hb E heterozygote/SEA	6	6	8.81 - 36.92
5. β-thalassemia heterozygote/ α-thalassemia 2	5	4	3.52 - 84.44
6. Hb E heterozygote/α-thalassemia 2	2	1	2.96, 83.21
7. β-thal heterozygote	8	4	<3.6
8. Hb E heterozygote	7	5	<4.3
9. α-thalassemia 2	4	1	3.0, 3.15, 3.73, 6.41

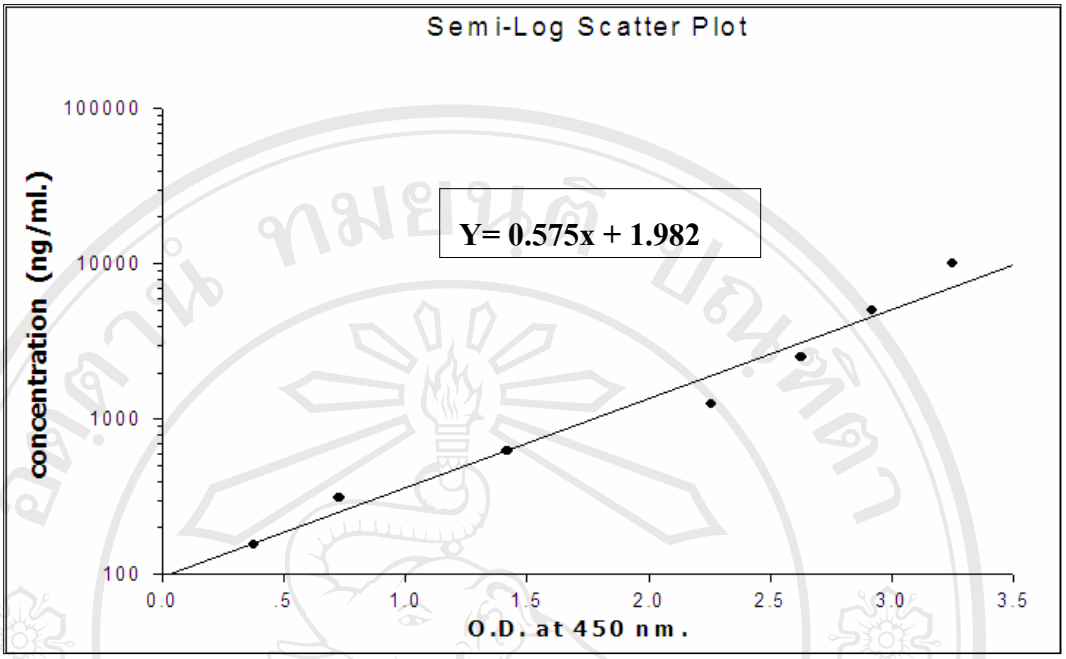

Figure 3.12 The representative standard curve of purified Hb Bart's concentrations was determined by developed sandwich ELISA. Purified Hb Bart's was serially diluted between 4.88 to 10,000 ng/ml as described in Materials and Methods. The reactivity was measured at O.D. 450 nm by spectrophotometer. The standard curve was plotted between Hb Bart's concentrations and O.D. 450 nm using sigma plot software.

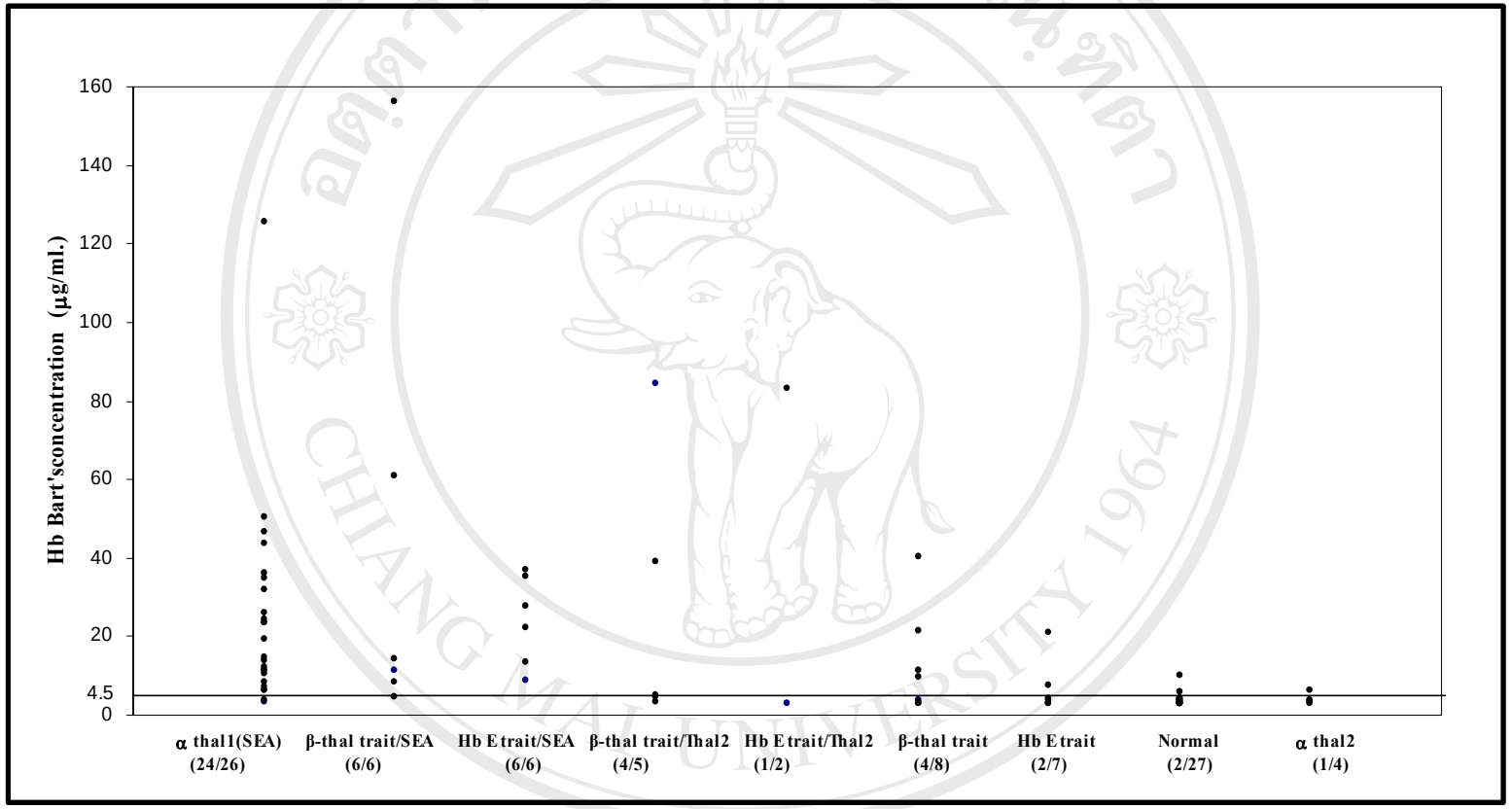

Figure 3.13 Comparison between PCR analysis and developed sandwich ELISA to identify α-thalassemia in 91 blood samples. The HPLC and PCR analysis was performed to categorize thalassemia types as described in Materials and Methods. Hb Bart's concentration was detected in all blood samples by developed sandwich ELISA. The Hb Bart's concentration was cut off at 4.5 µg/ml. Each dotted line represents individual sample.

3.5.1.8 Quantitation of hemoglobin Bart's concentration per gram of hemoglobin

For quantitative analyses of Hb Bart's in individual of α-thalassemia carriers, we calculated Hb Bart's concentrations and gram of hemoglobin. Mean ± SD of Hb Bart's concentrations per gram of hemoglobin of 25 normal samples were 25.18 ± 3.77. The confidential interval was between 17.64 to 32.72, 95% CI (17.64-32.72). Therefore, Hb Bart's concentration per gram hemoglobin was cut off at 33 μg/g Hb.

From these results confirmed that Hb Bart's concentrations per gram of hemoglobin in α-thalassemia group was higher than β-thalassemia heterozygote or Hb E heterozygote, especially in normal group. In group 2, Hb Bart's concentrations per gram hemoglobin from 24 α-thalassemia 1 (SEA type) were between 50.55 to 1,029.33 μg/g Hb. Among 6 double heterozygotes of β-thalassemia and α-thalassemia 1 with SEA deletion, the Hb Bart's concentrations per gram of hemoglobin were 33.09-1,419.95 μg/g Hb (group 3). Six samples with double heterozygotes of Hb E with SEA deletion (group 4) had Hb Bart's concentrations per gram hemoglobin of 66.78 to 315.51 μg/g Hb. In group 5, four samples of double heterozygotes of β-thalassemia and α-thalassemia 2 (3.7 kb deletion) had Hb Bart's concentrations per gram hemoglobin between 37.70 μg/g Hb to 620.88 μg/g Hb. The Hb Bart's concentrations per gram hemoglobin was detected at 682.04 μg/g Hb and 42.42 μg/g Hb in each one sample in group 6 and group 9 which is double heterozygotes of Hb E and α-thalassemia 2 and α-thalassemia 2 heterozygote, respectively. The remaining 32 samples were normal, β-thalassemia heterozygote and Hb E heterozygote in which Hb Bart's concentrations per gram hemoglobin was less than 31.19 μg/g Hb (group 1, 7 and 8) as results shown in Table 3.3 and Figure 3.14.

Surprisingly, 3, 4 samples and 3 samples of normal, β-thalassemia heterozygote and Hb E heterozygote had Hb Bart's concentrations per gram hemoglobin higher than 33 μg/g Hb.

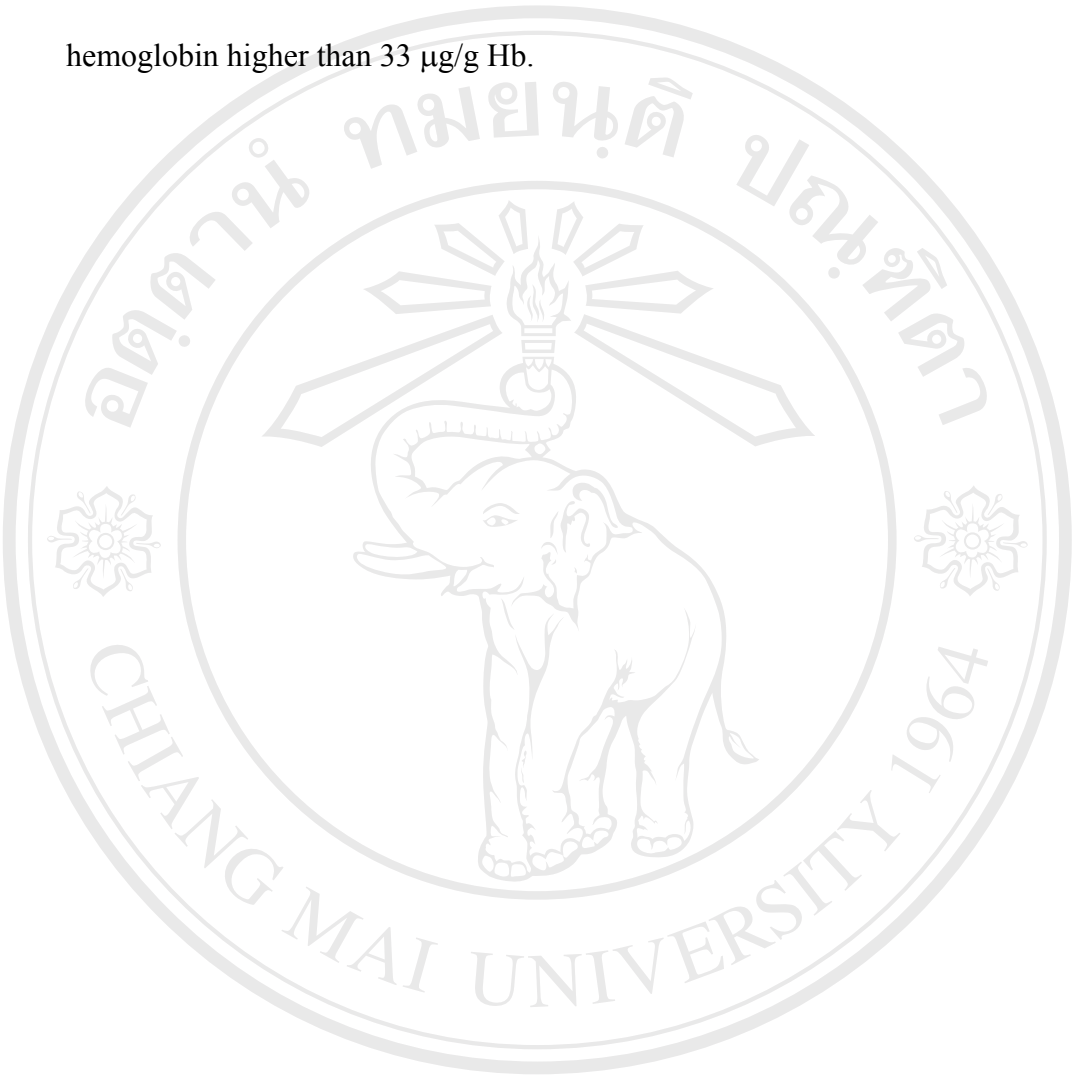

Table 3.3 The results of developed sandwich ELISA for Hb Bart's per gram hemoglobin in different categories

Category	Method		Hb Bart's concentrations (μg/g Hb)
	Standard (PCR)	Developed (ELISA)	
1. Normal	27	24	<31.19
2. α- thalassemia 1 (SEA type)	26	24	23.07-1,029.30
3. β-thalassemia heterozygote/SEA	6	6	33.09 – 1,419.95
4. Hb E heterozygote/SEA	6	6	66.78 – 315.51
5. β-thalassemia heterozygote/ α-thalassemia 2	5	4	27.68-620.88
6. Hb E heterozygote/α-thalassemia 2	2	1	24.06, 682.04
7. β-thal heterozygote	8	4	<21.68
8. Hb E heterozygote	7	4	<22.46
9. α-thalassemia 2	4	1	17.61, 24.35, 24.63, 42.42

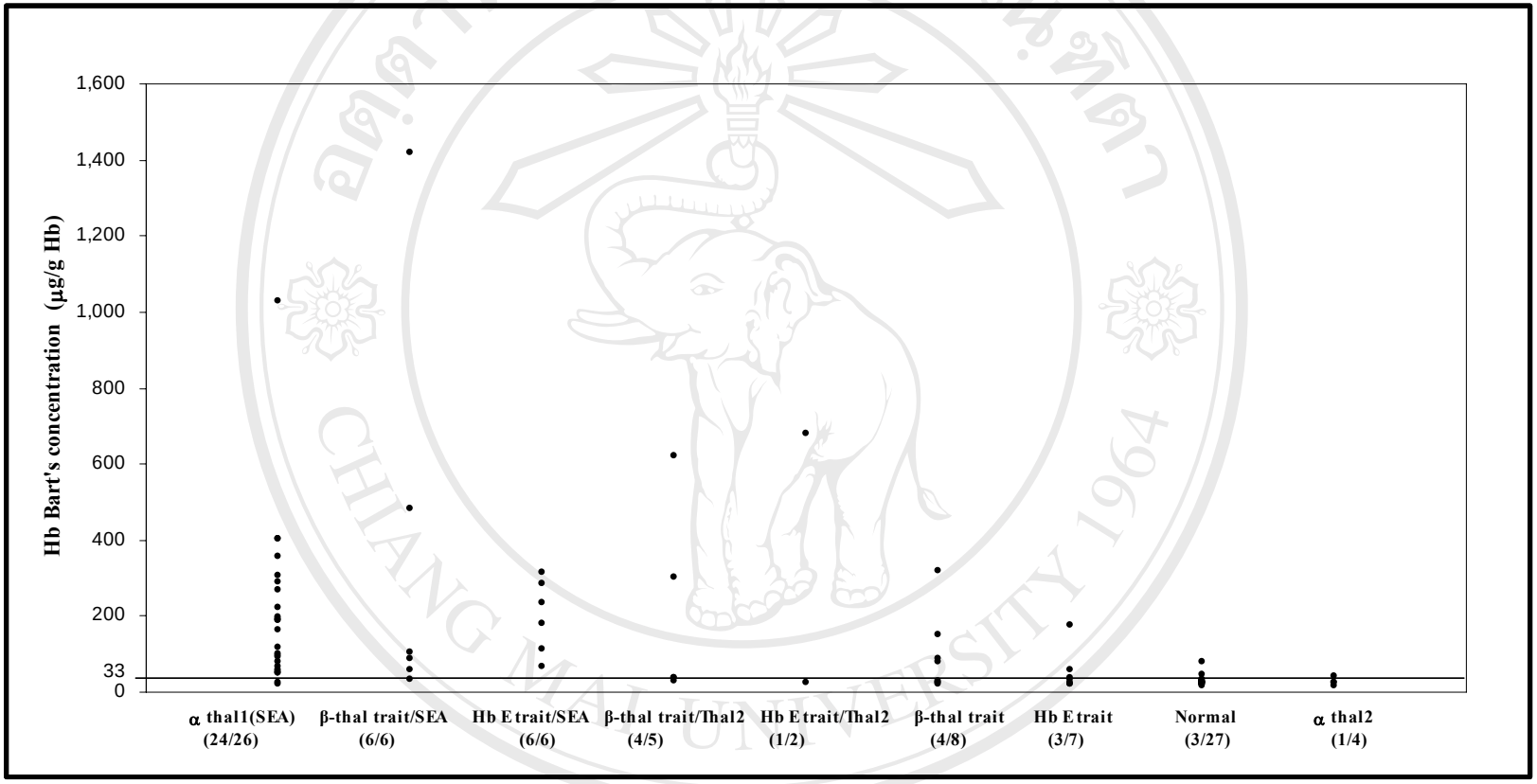

Figure 3.14 Quantitation of Hb Bart's concentration in total hemoglobin concentration to identify α-thalassemia in 91 blood samples. Hb Bart's concentration per gram hemoglobin was calculated in all blood samples in different category. The Hb Bart's concentration in total hemoglobin was cut off at 33 µg/g Hb. Each dotted line represents individual sample.